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# Absence of peripheral blood mononuclear cells priming in hemodialysis patients

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## Abstract

As a consequence of the proinflammatory environment occurring in dialytic patients, cytokine overproduction has been implicated in hemodialysis co-morbidity. However, there are discrepancies among the various studies that have analyzed TNF-α synthesis and the presence of peripheral blood mononuclear cell (PBMC) priming in this clinical setting. We measured bioactive cytokine by the L929 cell bioassay, and evaluated PBMC TNF-α production by 32 hemodialysis patients (HP) and 51 controls. No difference in TNF-α secretion was observed between controls and HP (859 ± 141 *vs* 697 ± 130 U/$10^6$ cells). Lipopolysaccharide (5 µg/ml) did not induce any further TNF-α release, showing no PBMC priming. Paraformaldehyde-fixed HP PBMC were not cytotoxic to L929 cells, suggesting the absence of membrane-anchored TNF-α. Cycloheximide inhibited PBMC cytotoxicity in HP and controls, indicating lack of a PBMC TNF-α pool, and dependence on *de novo* cytokine synthesis. Actinomycin D reduced TNF-α production in HP, but had no effect on controls. Therefore, our data imply that TNF-α production is an intrinsic activity of normal PBMC and is not altered in HP. Moreover, TNF-α is a product of *de novo* synthesis by PBMC and is not constitutively expressed on HP cell membranes. The effect of actinomycin D suggests a putative tighter control of TNF-α mRNA turnover in HP. This increased dependence on TNF-α RNA transcription in HP may reflect an adaptive response to hemodialysis stimuli.

**Key words**
- Cytokine
- Stress response
- Translational control
- Transcriptional blockage
- Priming
- Membrane-anchored tumor necrosis factor

## Introduction

During hemodialysis, blood contact with the dialysis membrane and other foreign surfaces promotes a range of complex and interconnected events, leading to an acute inflammatory response. Specifically, mononuclear cells and complement activation induce the secretion of a variety of inflammatory mediators including cytokines, reactive oxygen species, and nitric oxide. Therefore, hemodialysis affects several homeostatic systems and generates a complex of acute and chronic side effects also known as "bio-incompatibility phenomena" (1,2).

Secretion of cytokines by peripheral blood mononuclear cells (PBMC) has been implicated in the pathogenesis of dialysis-related

morbidity (3). In particular, tumor necrosis factor α (TNF-α) is a potent proinflammatory cytokine produced largely by macrophages in response to stimuli such as lipopolysaccharide (LPS), and binds to receptors present on virtually all cells (4,5). The physiological TNF-α range of action is broad and this cytokine can be studied as a model of cytokine response in hemodialysis patients. Acute secretion of TNF-α induced by hemodialysis is not well established, but its persistent low-level production may contribute to the chronic inflammatory response observed in end-stage renal disease patients (2,6). Bone reabsorption (3,7), anemia (8,9) and wasting (10,11) may all, in some measure, be attributable to TNF-α.

In hemodialysis, cytokine generation is presumed to take place in two steps: induction of mRNA transcription for cytokines by C5a and direct membrane contact, followed by LPS-induced translation of mRNA (priming/second signal theory; 12). However, the *in vitro* conditions on which this theory was based differed markedly from clinical dialysis. To test this postulate for routine hemodialysis, we evaluated whether a proinflammatory environment, as we see in hemodialysis patients, induces a priming of PBMC, with consequent higher production of TNF-α in response to a secondary stimulus (13). This immunological phenomenon was analyzed by cytotoxicity bioassay on L929 tumorigenic fibroblasts, a cell line specifically sensitive to TNF-α (14). In addition, we characterized and contrasted some aspects of TNF-α synthesis control in healthy individuals and hemodialysis patients.

## Patients and Methods

Blood samples were collected from 51 healthy controls from the blood donor center of Hospital São Paulo and 32 patients (36 ± 3 years old) in the chronic hemodialysis program. All patients were in the hemodialysis program of the Renal Division, Escola Paulista de Medicina, Universidade Federal de São Paulo (UNIFESP). The average time of dialytic treatment was 37 ± 7 months, using a regenerated cellulose membrane (cuprophane). Samples from hemodialysis patients were collected 48 h after the hemodialysis procedure. In both groups 5 ml of blood was drawn into a tube with heparin and kept on ice and the bioassay was performed 2 h later.

### Isolation of human peripheral blood mononuclear cells

PBMC were harvested as previously described (15). Briefly, each 5-ml blood sample was diluted with 5 ml of pyrogen-free normal saline and underlayered with 10 ml of Ficoll-Hypaque. The tube was spun at 450 *g* for 45 min at room temperature and the PBMC layer was harvested, washed in saline and centrifuged at 400 *g* for 10 min. Then, cells were washed in saline two additional times and resuspended in filtered tissue culture medium (RPMI 1640, pH 7.4; Sigma, St. Louis, MO, USA), containing 1 mM folic acid, 23 mM L-asparagine, 0.2 mM glutamine, 0.1 mM pyruvic acid, 100 U/ml penicillin, 200 µg/ml streptomycin, 10 mM HEPES, and 5% fetal bovine serum (Sigma). PBMC were counted using a standard hemocytometer and a suspension of $6 \times 10^6$ PBMC/ml was prepared in RPMI.

### *In vitro* production of TNF-α by peripheral blood mononuclear cells

A modified TNF-α bioassay was used (16). L929 tumorigenic murine cells (ATCC) specifically sensitive to TNF-α (14) were cultivated in RPMI 1640, pH 7.4, containing 1 mM folic acid, 23 mM L-asparagine, 0.2 mM glutamine, 0.1 mM pyruvic acid, 100 U/ml penicillin, 200 µg/ml streptomycin, 10 mM HEPES, and 5% fetal bovine serum. For the cytotoxicity assay, L929 cells were seeded into flat bottom 96-well plates at a density of

5.5 x $10^4$ cells per well and incubated overnight at 37ºC in a 5% $CO_2$ atmosphere. After incubation, spent medium was removed and 100 µl of serial dilutions of PBMC suspensions (1.0 x $10^3$ to 3.2 x $10^4$ PBMC/well) was added to each well. After 4 h, 10 µl of medium containing actinomycin D was added to each well, yielding a final concentration of 5 µg/ml. In a special set of experiments actinomycin D was added immediately after PBMC seeding over L929 cells. Plates were similarly re-incubated for 20 h and viable L929 cells were stained with 20 µl/well of 0.75% crystal violet in 30% acetic acid for 15 min, rinsed and dried. Methanol was added to solubilize the stained cells, and the absorbance of each well was read at 630 nm with a Vmax-Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Percent cytotoxicity was calculated by reference to control monolayers incubated in medium only. One cytotoxic unit was defined as the number of cells that killed 50% of the L929 cells. Results are reported as cytotoxic units per $10^6$ PBMC plated.

### Fixation of peripheral blood mononuclear cells with paraformaldehyde

PBMC were incubated with 1% paraformaldehyde for 5 min at room temperature. Thereafter, PBMC were washed three times in RPMI 1640, counted and resuspended in RPMI 1640. Fixed PBMC were then used in the TNF-α cytotoxicity assay.

### Treatment with cycloheximide

In the cytotoxicity assay, L929 cells were seeded and incubated overnight at 37ºC in a 5% $CO_2$ atmosphere. After incubation, spent medium was removed and 100 µl of serial dilutions of PBMC suspensions was added to each well. Immediately after, 10 µl of medium containing cycloheximide was added to each well, yielding a final concentration of 10 µg/ml. After 4 h, 10 µl of medium containing actinomycin D was added to each well, yielding a final concentration of 5 µg/ml. Of note, as positive control recombinant TNF-α was added to a subset of wells.

### Statistical analysis

Statistical analysis was performed using the *t*-test and the Bonferroni multiple comparisons test when appropriate. All statistical analyses were performed using the StatView Software (Abacus Concepts, Inc., Berkeley, CA, USA, 1996). Data are reported as mean ± SEM and the level of significance was set at $P<0.05$.

## Results

For measurement of cytokine PBMC production we used an *in vitro* cell cytotoxicity assay for TNF-α which is simple and sensitive. In contrast to previous studies that used ELISA, the evaluation of bioactive TNF-α production by PBMC of healthy control individuals and patients on chronic hemodialysis did not show any difference between the two groups (859 ± 141 *vs* 697 ± 130 U TNF-α/$10^6$ cells, P = 0.76) (Figure 1). Of note, none of the individuals enrolled in this study presented any pathology at the time of blood sample drawing or were taking any medication that could markedly affect TNF-α secretion or activity. Concomitantly to the cell cytotoxicity assay, a neutralizing assay with a monoclonal anti-TNF-α antibody (Innogenetics S.A., Ghent, Belgium) was performed. This experiment showed a strong inhibition of L929 cell cytotoxicity induced by PBMC from control individuals or hemodialysis patients, suggesting that the observed biological phenomenon was mediated by TNF-α secretion in the cell culture medium.

The bacterial-derived LPS is a strong stimulus for TNF-α production and secretion. Thus, we added 5 µg/ml of LPS to the cell culture medium used in the L929 cell

cytotoxicity assay as an additional stimulus of TNF-α production by PBMC. Surprisingly, we observed no increase in TNF-α production by PBMC from 25 controls (974 ± 217 *vs* 1018 ± 188 U TNF-α/$10^6$ cells, P = 0.49) or by PBMC from hemodialysis patients (776 ± 211 *vs* 561 ± 122 U TNF-α/$10^6$ cells, P = 0.47). Of note, LPS (5 µg/ml) by itself was incapable to induce L929 cell death.

The presence of cell membrane-anchored TNF-α was studied by paraformaldehyde fixation of PBMC (17) from control individuals and hemodialysis patients, immediately before PBMC seeding over L929 cells. The fixation process completely abolished the L929 cell death induced by the treated PBMC from six control individuals and five hemodialysis patients.

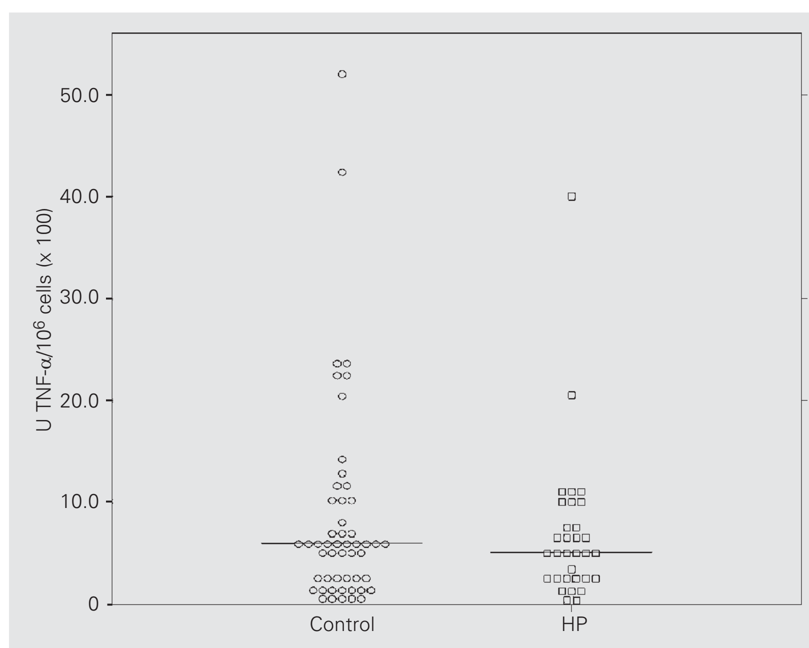


Figure 1. TNF-α production by peripheral blood mononuclear cells from 51 healthy controls and 32 hemodialysis patients (HP). Samples from hemodialysis patients were collected 48 h after the dialytic procedure. All the patients were on routine hemodialysis using regenerated cellulose membranes (cuprophane). TNF-α was measured with a cytotoxicity assay using specifically TNF-α-sensitive L929 tumorigenic murine fibroblasts. The horizontal line is the median (P = 0.76, two-tailed Mann-Whitney U-test).

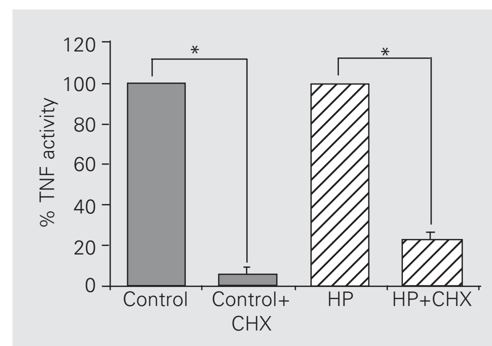


Figure 2. Effect of cycloheximide (CHX) on TNF-α production by peripheral blood mononuclear cells measured with the L929 cell bioassay in the absence and presence of 10 µg/ml cycloheximide. The addition of the translation blocker cycloheximide statistically reduced TNF-α production in healthy controls and hemodialysis patients (HP). *P<0.01 compared to respective control (*t*-test).

*De novo* protein synthesis plays an important role in the secretion of this cytokine. Consequently, we examined whether inhibition of protein synthesis affected PBMC-induced L929 cell death. As shown in Figure 2, cycloheximide abolished TNF-α secretion by PBMC from nine control individuals and 14 hemodialysis patients. As a positive control, recombinant TNF-α was added to a subset of wells. These data suggest that there is no TNF-α storage in the PBMC from hemodialysis patients and that cycloheximide did not interfere with the L929 cell death process.

Cytokine mRNA stability is an important step in the control of these monokines, and we evaluated the TNF-α mRNA stability in PBMC from healthy controls and hemodialysis patients by adding the transcription blocker actinomycin D at two different time points, i.e., immediately and 4 h after PBMC seeding. As shown in Figure 3, there was no difference in TNF-α secretion by healthy control PBMC (388 ± 76 *vs* 263 ± 75 U TNF-α/$10^6$ cells) when actinomycin was administered at the two distinct time points. In contrast, we observed a significant reduction of TNF-α production by PBMC from hemodialysis patients (588 ± 92 *vs* 264 ± 44 U TNF-α/$10^6$ cells, P = 0.007) in the presence of actinomycin D since the beginning of the bioassay. These data suggest a lower TNF-α mRNA stability in hemodialysis patients, reflecting a putative tighter control of TNF-α synthesis.

## Discussion

The ability of cells to adopt an increased

functional status under certain conditions or in a defined environment is referred to as priming. This phenomenon was originally studied in macrophages, but has also been observed in neutrophils, basophils, lymphocytes and eosinophils. In addition, priming is achieved by the action of soluble mediators, although other factors such as the presence of co-secreted mediators, adhesive interactions with neighboring cells or with intercellular matrix proteins might also play a role (13). In hemodialysis, blood-dialyzer interaction and uremia establish a chronic proinflammatory environment (2,18-21), potentially inducing immunological priming. In addition, previous studies have shown that patients undergoing hemodialysis via routine regenerated cellulose membranes present evidence of elevated IL-1 and TNF before a dialysis session and a further increase at the end of the procedure. However, not all studies have confirmed these findings (2). In this study we analyzed the presence of PBMC priming through the evaluation of TNF-α synthesis as a model of cytokine response to the uremic environment. For this purpose, we used a TNF-α bioassay that detects the activity of this cytokine by specific cell death of L929 tumorigenic murine fibroblasts (14).

Measurement of plasma TNF-α levels in hemodialysis patients can be performed by immunoassays, molecular biological techniques and bioassays. Immunoassays and molecular biological techniques are highly specific and are not influenced by cytokine-binding proteins and inhibitors. Hence, they are very useful in detecting cellular cytokine production (22). In contrast, bioassays are less sensitive and are affected by soluble receptors. Moreover, analysis of PBMC is more likely to yield consistent results regarding increased cytokine production during hemodialysis treatment than circulating plasma concentrations (23). In this context, we studied PBMC from 51 healthy controls and 32 hemodialysis patients. We did not observe differences between controls and hemodialysis patients in bioactive TNF-α production. Also, the addition of LPS, a condition that can occur *in vivo* by back-transport from the dialysate (1,24,25), did not increase TNF-α production in either group. These data suggest that bioactive TNF-α production is not enhanced in hemodialysis patients and that there is no priming of these cells in spite of the multiple stimulus environment found in uremic-hemodialysis patients.

The gene for human TNF-α encodes a pro-hormone that is inserted into the cell membrane as a polypeptide. This membrane-bound form of TNF-α is bioactive and has been implicated in the paracrine activities of TNF-α in tissues (4,26). Cleavage by TNF-α-converting enzyme, a member of the disintegrin and metalloprotease family, generates the soluble forms of TNF (26). The secreted form of TNF-α (27) has been studied in hemodialysis patients, but there is no information about the membrane cytokine form. To analyze the expression of membrane-anchored TNF-α, PBMC were previously fixed with paraformaldehyde (17) and seeded over L929 cells. This process abolished L929 cell death in both groups, suggesting that there is no constitutive expression of membrane-anchored TNF-α in chronic hemodialysis patients, despite the continuous stimuli to which they are exposed.

The production of cytokines and expression of cytokine receptors are under tight and complex biological control, including

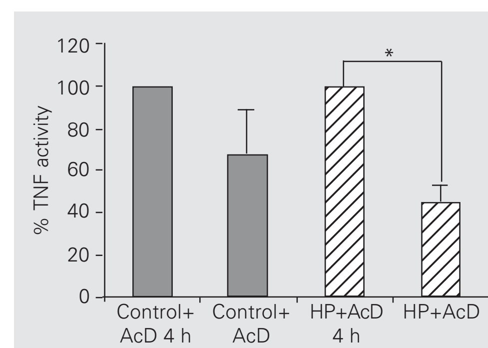


Figure 3. Effect of actinomycin D (AcD) on TNF-α production by peripheral blood mononuclear cells (PBMC) measured by the L929 bioassay in the absence and presence of 2 µg/ml actinomycin D, 4 h or immediately after PBMC seeding over L929 cells. Addition of the transcription blocker actinomycin D at the beginning of the experiment did not affect TNF-α production in healthy control PBMC but significantly reduced it in hemodialysis patients (HP). *P<0.05 (*t*-test).

negative and positive feedback by the cytokines themselves (28). In general, most cytokine genes are not expressed, at least at the translational level, unless specifically stimulated by noxious events (29). To analyze the presence of pre-formed TNF-α pro-hormone stored intracellularly in hemodialysis patients, PBMC were treated with the translation blocker cycloheximide and submitted to a bioassay. The protein synthesis blockage abolished TNF-α-induced L929 cell death in both groups. These data imply that there is no TNF-α pro-hormone pool in patients on hemodialysis treatment, corroborating the importance of *de novo* protein synthesis in cytokine production control and the absence of an alternative cell priming response.

The mRNAs of transiently expressed genes frequently contain an AU-rich sequence in the 3'-untranslated region. This nucleotide sequence confers high mRNA instability, establishing another control of cytokine biosynthesis (30). To examine TNF-α mRNA stability in hemodialysis patients, PBMC were treated with the transcriptional blocker actinomycin D. Pretreatment with actinomycin D had no effect on healthy control cells, but caused a significant reduction in bioactive TNF-α production by PBMC from hemodialysis patients. Therefore, it seems that TNF-α mRNA from hemodialysis patients is produced at a higher turnover rate depending on the transcription of new cytokine messages, suggesting a putative tighter control of cytokine synthesis in patients submitted to hemodialysis than in healthy controls.

The uremic medium is a harsh environment, where proinflammatory stimuli are present during the development of end-stage renal disease, as well as during the replacement of renal function with dialysis. As stress response peptides (28), cytokines are induced continuously in hemodialysis patients, determining an adaptive response of the entire organism. In this setting the host counteracts high cytokine levels by releasing soluble cytokine receptors or by synthesizing high-affinity anti-cytokine antibodies (31). This biological phenomenon occurs with TNF-α (1,32). Using a TNF-α bioassay it was possible to evaluate the synthesis and release of the bioactive cytokine (33-35). The present data suggest that there is no priming of PBMC and no constitutive expression of membrane-anchored TNF-α. In addition, we did not find a cellular pool of TNF-α pro-hormone, but we observed a putative tighter control of TNF-α mRNA stability. However, further experiments applying molecular biology techniques and protein expression analysis are necessary to confirm those findings. Despite the previously mentioned limitations, the present data imply that the concerted action of these multiple mechanisms may be necessary to avoid additional inadequate production of TNF-α with further derangement of normal homeostasis.

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